Influence of salinity on larval development of *Sicyopterus lagocephalus* (Pallas, 1770) (Gobioidei)

by

Céline ELLIEN* (1), Pierre VALADE (2), Jérôme BOSMANS (3a), Laura TAILLEBOIS (4),
Nils TEICHERT (3b) & Philippe KEITH (4)

ABSTRACT. - The widely distributed amphidromous goby *Sicyopterus lagocephalus* needs drastic change of habitat to fulfil its life-cycle: adults live and spawn in rivers, where eggs hatch in pro-larvae that have to reach the sea in a narrow temporal scale, to acquire marine characteristics, and begin their oceanic dispersal as planktonic larvae. Post-larvae return to rivers where they recruit and grow to the adult reproductive stage. Such a life-cycle raises the question of salinity changes between these different developmental stages. Through an experimental approach, we observed and described the chronology in the appearance of marine larvae characteristics, according to 5 different salinities ranging, from 0% to 100% seawater. It appears that *S. lagocephalus* needs an increase of salinity to acquire marine morphological characteristics, without being strict upon the salinity value itself. Whatever the tested salinity, the transformation of freshwater pro-larvae into marine larvae follows the same pattern within 48h: the yolk sac is absorbed until it only consists in the oil globule, pectoral fins develop and become functional, pigmentation spreads above the digestive tract, eyes become pigmented and functional, mouth and anus open, and the head appears more shaped. Pro-larvae behaviour evolves once in seawater: larvae remain on vertical position with their head oriented downwards (i.e., freshwater pro-larval behaviour) but they also spend more time horizontally, after the opening of the mouth, showing a behaviour of horizontal swimming, with acceleration phases, that may be interpreted as a hunting behaviour. Even a low salinity (i.e., less than 10% of seawater) is sufficient to induce these transformations, with the same timing than 100% seawater, leading to the conclusion that we did not underline a minimum salinity threshold for the acquisition of marine larvae morphological characteristics. Moreover, if the pro-larvae, after a first contact with brackish water, secondarily return to freshwater, their transformation is not stopped. However, pro-larvae that remain in 100% freshwater do not develop into marine larvae, neither in their morphological characteristics nor in their behaviour, and they die within 4 days.

RÉSUMÉ. - Influence de la salinité sur le développement larvaire de *Sicyopterus lagocephalus* (Gobioidei).

L'espèce amphidrome à large répartition *Sicyopterus lagocephalus* est confrontée à des changements drastiques de milieux pour accomplir son cycle de vie: les adultes vivent et pondent en rivière, après l'éclosion les pro-larves doivent atteindre très rapidement la mer pour acquérir les caractéristiques du stade larvaire planctonique et commencer leur dispersion marine. Les post-larves retournent ensuite vers les rivières où elles recrutent et grossissent jusqu'à l'état adulte. Un tel cycle de vie soulève la question des changements de milieu entre les différents stades de développement. Nous avons observé et décrit à l'aide d'expérimentations la chronologie de l'apparition des caractéristiques liées à la vie marine des larves, et ce, en fonction de 5 salinités différentes allant de 0 à 100% d'eau de mer. Il apparaît que *Sicyopterus lagocephalus* a besoin d'une augmentation de salinité, quelle qu'elle soit, pour acquérir les caractéristiques morphologiques liées à la vie marine. En effet, quelle que soit la salinité testée, la transformation des pro-larves d'eau douce en larves marines suit le même patron en 48 h : le sac vitellin se résorbe jusqu'à ne consister qu'en une gouttelette lipidique, les nageoires pectorales se développent et deviennent fonctionnelles, la pigmentation s'étend au-dessus du tube digestif, les yeux se pigmentent et deviennent fonctionnels, la bouche et l'anus s'ouvrent et la tête devient plus profilée. Le comportement des pro-larves évolue à partir du moment où elles sont dans l'eau de mer: les larves restent en position verticale avec la tête orientée vers le bas (i.e., comportement des pro-larves en eau douce), mais elles passent également plus de temps à l'horizontale, après l'ouverture de la bouche, présentant un comportement de nage sur l'horizontale, avec des accélérations, pouvant être interprétées comme un comportement de chasse. Même une faible variation de salinité (i.e., moins de 10% d'eau de mer) est suffisante pour induire cette transformation, avec la même chronologie qu'avec 100% d'eau de mer. Ceci nous permet de conclure que nous n'avons pas mis en évidence de seuil de salinité minimale pour l'acquisition des caractéristiques morphologiques liées à la vie marine. En outre, si la larve, après un premier contact avec l'eau salée, est remise en eau douce, sa transformation continue à se dérouler normalement. Cependant, les pro-larves qui restent en eau douce stricte n'évoluent pas en larves marines, que ce soit pour leurs caractéristiques morphologiques ou leur comportement. Elles meurent dans les 4 jours après leur éclosion.

Key words. - Larval development - Amphidromy - Salinity range - *Sicyopterus lagocephalus* - Larval morphology - Reunion Island.

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(1) Université Pierre et Marie Curie, UMR 7208 (MNHN-CNRS-UPMC- IRD), Département milieux et peuplements aquatiques, Muséum national d'Histoire naturelle, 43 rue Cuvier, CP26, 75231 Paris CEDEX 05, France. [cellien@mnhn.fr]

(2) OC..E.A. Consult', BP 22, Centre régional d'application aquacole, 97427 Étang Salé, La Réunion, France. [valadeoceaconsult@zeop.re]

(3a) ARDA, Port Ouest, Hangar 10, 97420 Le Port, La Réunion, France. [bosmansj.arda@orange.fr]

(3b) ARDA, ZI Les sables, BP 16, 97427 Étang Salé, La Réunion, France. [teichert.arda@orange.fr]

(4) MNHN, UMR 7208 (MNHN-CNRS-UPMC- IRD), Département milieux et peuplements aquatiques, Muséum national d'Histoire naturelle, 43 rue Cuvier, CP26, 75231 Paris CEDEX 05, France. [taillebois@mnhn.fr] [keith@mnhn.fr]

* Corresponding author

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Rivers on tropical volcanic islands are mainly colonised by diadromous species, including fishes, crustaceans and molluscs. Such a life cycle is indeed the best adapted to the particular conditions in these habitats, namely young oligotrophic rivers, characterised by extreme climatic and hydrological variations on a seasonal scale (Keith, 2003; Keith *et al.*, 2005a).

Amphidromy, as defined by McDowall (1988, 1992) is a type of diadromy in which organisms spend a part of their life-cycle in freshwater and the other part in seawater, but the migration between these two systems is not related to the reproductive event. Following this definition, *Sicyopterus lagocephalus* (Pallas, 1770) (Teleostei: Gobiidae: Sicydiinae) is an amphidromous goby, characterised by adults living and spawning in freshwater. After hatching, the pro-larvae (or free embryos) drift downstream to the sea, where they transform into planktonic larvae which undergo a dispersive phase through oceanic currents. Then, post-larvae return to the rivers, where they metamorphose into juveniles (Keith *et al.*, 2008; Taillebois *et al.*, 2011), and grow until the reproductive adult stage. Thus, *S. lagocephalus*, like all amphidromous species, has the particularity to shift between different environments during its life-cycle, and that implies great physiological adaptative abilities.

In insular rivers, the diversity of fish communities and then biodiversity, relies mainly on amphidromous gobies, at least for the Caribbean and Indo-Pacific islands, and most of these species are endemic (Lim *et al.,* 2002; Keith, 2003; Marquet *et al.*, 2003; Keith *et al.*, 2006; Lord and Keith, 2008; Keith et Lord, 2011). Among these amphidromous gobies, *S. lagocephalus* is widely distributed throughout the Indo-Pacific systems, being probably the Sicydiinae with the largest spatial distribution (Keith, 2003; Keith *et al.*, 2005a). It occurs throughout the Indian Ocean, from the Comoros and Mascarene islands to Indonesia. In the Pacific Ocean, *S. lagocephalus* distribution area spreads from Indonesia, to Society Islands (French Polynesia) through Japan, Australia and New Caledonia (Keith *et al.*, 2005a).

Besides their importance regarding their strong contribution to river fauna biodiversity (at least for the Caribbean and Indo-Pacific islands), one of the main interests of Sicydiinae species consists in their high economic value. Indeed, some species of the genera *Sicydium* (for Caribbean systems) and *Sicyopterus* (for Indo-Pacific systems) are targeted by traditional intensive fisheries, at their post-larval stage, when they migrate back to rivers. They constitute an important source of food for local human populations, leading to a substantial stock depletion (Keith *et al.*, 2002; Keith, 2003).

In this context, the biology and ecology of these species need to be better understood, to propose management and conservation plans, to preserve both stocks and biodiversity.

Within the framework of this study, we focus on *S. lagocephalus* in Reunion Island (Mascarene Archipelagos, Indian Ocean), where it is particularly subject to intense fisheries. In this region, this species has been studied in its freshwater phase, so that the pro-larval as well as post-larval (i.e., "bichiques"), juveniles and adult stages have been observed and described (Keith *et al.,* 2008; Valade *et al.*, 2009). The reproduction patterns have also been described (Henaff, 2008; Teichert, 2009). Between the pro-larval and the juvenile stages, that both take place in freshwater, larvae disperse in the ocean. This marine phase remains quite unknown, whereas it is particularly interesting, 1) in an ecological point of view, as it allows large scale dispersal and explains the distribution pattern of the species, and 2) in a physiological point of view, as it implies two transitions between fresh and sea-water. Valade *et al.* (2009) focused on *S. lagocephalus* larval development from hatching to their migration to the sea, and they described the different larval stages from L1a (i.e., pro-larvae: freshwater stage) to L2 (i.e., marine larvae with opened mouth and no more yolk sac). They showed that freshwater pro-larvae do not undergo any development in freshwater, and that it is their arrival at sea that triggers their morphological transformation. Their results also highlighted that larval development is quicker when the temperature is high and that it varies according to water depth during the downstream migration. The purpose of our study is to bring new insights into the transition between freshwater and ocean, and to describe the acquisition of marine larvae morphological characteristics, as we observed some divergences with Valade *et al.* (2009). To assess the influence of salinity on larval transformation, we observed larval development in a low salinity tank to compare their development to higher salinity environments. This study is considered as a first step in the understanding of *S. lagocephalus* marine phase and further developments are envisaged.

MATERIAL AND METHODS

Review on biological model

In Reunion Island, *S. lagocephalus* freshwater stages have been studied; it is well known that this species lives in rivers with moderate to strong currents, clear and well oxygenated water (Keith, 2003; Keith *et al.*, 2008).

A female spawns between 10000 and 100000 small eggs (Delacroix and Champeau, 1992), that are laid on the underside of rocks and pebbles. The male takes care of the clutch (Keith, 2003), until the eggs hatch into pro-larvae ± 48 h after fertilization (Delacroix and Champeau, 1992). Spawning period spreads from January to June, coinciding with summer and autumn in the southern hemisphere, i.e., during the warmest but also the rainy season. Valade *et al.* (2009) show that river temperature has a significant effect on pro-larvae survival. They empirically defined optimal temperatures (20-23°C) for pro-larvae survival. These temperatures correspond to mean daily temperature in La Reunion rivers during *S. lagocephalus* reproduction period. Moreover, strong rainfalls may favour strong currents in the rivers and consequently a better oxygenation of the clutches and a rapid transport of the pro-larvae to the sea (Valade, 2001). Valade (2001) has also shown that, according to the rivers length (i.e., 36 km maximum) and the river flow intensity, pro-larvae are transported downstream to the sea in less than 24 h, whatever the clutches' location in the river. During this transport, the pro-larvae, even if they are not able to resist to the river flow, are not totally passive: alternatively, they actively swim towards the surface, then cease swimming and sink. This vertical behaviour has been observed both in the field (Keith, 2003), and in experimental tanks within the present study (see also Teichert, 2009) as well as on *Sicyopterus japonicus* pro-larvae (Iida *et al*., 2010). This behaviour facilitates the transport of the pro-larvae to the sea by maintaining them on the surface where the current flows faster. Valade *et al*. (2009) show that the water depth has a significant effect on pro-larvae survival; the deeper the river, higher the mortality. This depth-factor is explained by the vertical oscillation of pro-larvae: in shallow water, this vertical oscillation requires less energy compared to greater water mass.

Once in the sea, the pro-larvae turn into marine larvae, which are dispersed by the oceanic currents during 4 to 9 months (Lord *et al.,* 2010; Lord *et al*., in press). Such a long dispersive period, along with an important variability in the Pelagic Larval Duration (PLD) at the annual scale (Henaff, 2008; Lord *et al.,* 2010) allow the colonisation of distant habitats and could explain the large distribution of the species at the Indo-Pacific scale (Keith *et al*., 2005a; Lord *et al*., 2010). At the end of the marine dispersive stage, larvae have to find freshwater systems to settle as post-larvae (Keith, 2003). They undergo a metamorphosis allowing them to live, grow and mature in freshwater as benthic juveniles (Keith *et al*., 2008; Taillebois *et al.,* 2011).

Valade *et al*. (2009) described five larval stages observed for *S. lagocephalus,* at sea, without feeding and for a water temperature between 20 and 23°C. The L1 stage comprises 4 stages that Valade *et al*. (2009) defined as free-embryos: L1a stage, defined as larvae at hatching (i.e., no trace of eye, no sign of jaw, not pigmented, yolk sac at its maximum size: river stage); L1b stage, defined as larvae just arriving at sea, with 0-20 hours spent in sea water (i.e., early eye (lens present, retina not pigmented), eyes on lateral sides of the head, posterior tip of mandible detectable as a prominence below the eye, body not pigmented, beginning of yolk sac absorption: marine stage); L1c stage, defined as larvae with 20-40 hours spent in sea water (i.e., lens present and pigment beginning to appear on retina, beginning of the migration of eyes in anterolateral position of head, mouth is formed but closed, appearance of pectoral fins, multiplication of chromatophores on the body: marine stage); L1d stage, defined as larvae with 40-65 hours spent in sea water (i.e., lens pigmented, eyes in anterolateral position of head, mouth open in sub-inferior position, pectoral fins developed, appearance of chromatophores in the cephalic area: marine stage). The last stage corresponds to L2 stage, with larvae spending more than 65 hours in sea water (i.e., mouth open in terminal position and operating, ability to intake and digest exogenous food, no more yolk sac, many chromatophores in the cephalic area and along the body, all internal organs in place: marine stage).

Sampling protocol and rearing condition

S. lagocephalus egg clutches were collected at the end of April 2010, in the lower reaches of Langevin River (southwest of Reunion Island, Mascarene Archipelagos, Indian Ocean), at 800 m of the river mouth. This river is characterised by an average flow of 1.18 $m^3.s^{-1}$ (Teichert, 2009). As the egg clutches are stuck on the inferior face of the rocks embedded in sand and gravels on the bottom of the river, we had to turn the rocks one after the other to find them. The newly laid eggs are white, and turn grey when they are ready to hatch; we therefore collected only grey clutches. Thus, four grey clutches were collected and immediately transferred into two buckets filled with the water of the river, where they all immediately hatched after the transfer. Even if one egg clutch produces enough pro-larvae to fulfil the experiment, mixing several clutches allow to reduce the risk of failure (e.g., larvae malformation leading to strong mortality) linked to a genetic problem on one particular clutch.

The pro-larvae were transported in the buckets to the laboratory within 3 h after their collect. Once in the laboratory, they were randomly distributed between two 45 l tanks (i.e., 40 x 38 x 29 cm), filled with freshwater (tap water) that was first oxygenated during 48 h before receiving the pro-larvae. The pro-larvae were maintained in these tanks during 48 h before starting the experiment, the freshwater of the 2 tanks being entirely renewed in 24 h, with tap water previously oxygenated during 24 h. During this storing phase, pro-larvae density reached, on average, 500 $ind.l^{-1}$

Experimental protocol

Influence of larval density

To assess the importance of larval density at the beginning of the experiment (i.e., when pro-larvae are transferred in tanks filled with seawater), an experimental study has been conducted: 4 densities (150 $larvae.l^{-1}$, 100 $larvae.l^{-1}$, 50 $larvae.l^{-1}$, 30 $larvae.l^{-1}$) were tested, with duplicates, so that 8 tanks of ± 45 l (i.e., 40 x 38 x 29 cm) were installed. Each tank was filled up with off shore drilling water circulating in open circuit, also passing through the two degassing columns before filling up the tanks. Aeration took place directly in the tank. Larvae were not fed during the whole experiment. During the experiment, the temperatures were on average 27,1°C (see below for detailed conditions and daily logistic).

Influence of salinity

To assess if a low salinity is sufficient to stimulate the acquisition of marine larvae morphological characteristics and then the passage from the pro-larval to the larval stage, a range of salinities was set up. Five salinities were tested, with duplicates, so that 10 tanks of ± 45 l (i.e., 40 x 38 x 29 cm) were installed:

- 2 tanks filled with 100% tap water that was first oxygenated during 24 h. The freshwater was renewed by quarter with tap water (previously oxygenated during 24 h), i.e., ± 11 l of clean water was poured in the tanks every 3 h during the day-light period of 12 h, so that the water in the tanks was entirely renewed each day;
- 2 tanks filled with 25% sea water (i.e., drilling water from offshore, passing through 2 degassing columns and oxygenated before filling up the tanks) and 75% freshwater (i.e., tap water that was first oxygenated during 24 h). The water of the tank is renewed by quarters entirely in 12 h, conserving the same proportions between fresh- and sea-water;
- 2 tanks filled with 50% sea water and 50% freshwater (note that the same protocol than previously described is applied, to renew daily the water of the tanks);
- 2 tanks filled with 75% sea water and 25% freshwater;
- 2 tanks filled with 100% seawater: in that case, the off shore drilling water circulated in open circuit, also passing through the two degassing columns before filling up the tanks. Aeration took place directly in the tank.

In each tank, the pro-larvae density at the beginning of the experiment was 100 ind.l^{-1}.

Larvae were not fed during the whole experiment. During the experiment, the temperatures were on average: 26,9°C for the freshwater and 27,1°C for the seawater. All tanks were located under electric light with a programmed 12 h day/night cycle that reproduced natural diary cycle in La Reunion Island. Each day, the tanks were cleaned up by siphoning off the dead individuals and waste material. The mortality rate in the different tanks has not been assessed quantitatively only qualitatively.

Pro-larvae and larvae were randomly sampled every 1 h 30 on a period of 10 hours per day and observed under a binocular lens set with a camera (Olympus C-5050; optical zoom x3; Olympus Ltd, Paris) to characterise morphological development in strict fresh and seawater, and once a day to test salinity effects.

48h after the beginning of the experiment (i.e., 4 days after hatching), a batch of pro-larvae was transferred from their initial freshwater tank to another tank filled with freshwater previously oxygenated during 24 h. 3l of seawater were poured in the tank of freshwater (corresponding to a proportion of 6.67% of seawater, that is to say a weaker salinity than those tested in the main part of the experiment). Pro-larvae were maintained 24 h in this brackish water. Then *S. lagocephalus* individuals were transferred again in a tank filled with 100% freshwater previously oxygenated. Morphological characteristics of these individuals were observed

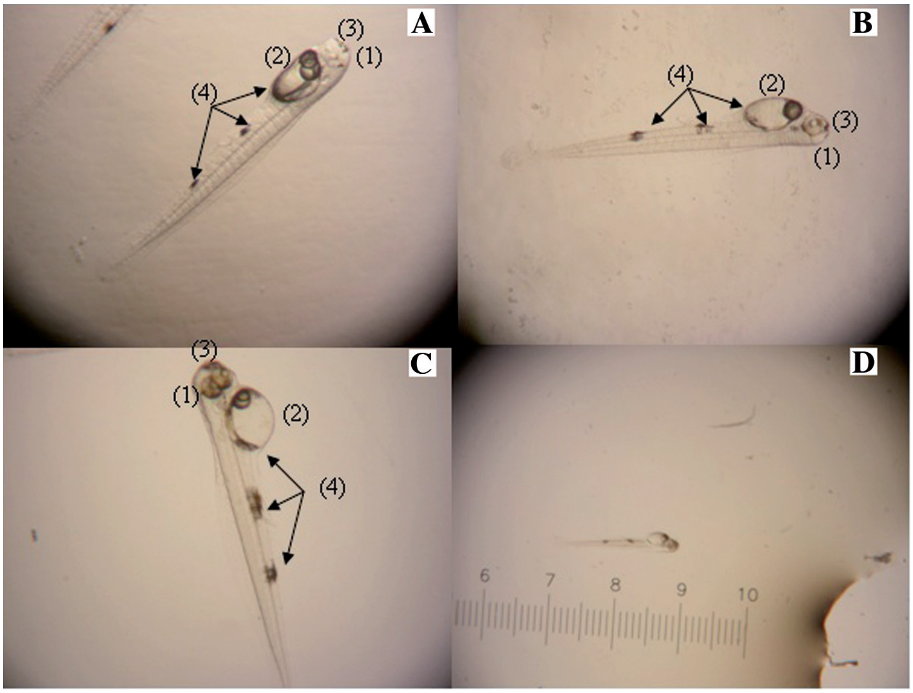

Figure 1. - Standard morphological development of *Sicyopterus lagocephalus* pro-larvae, in freshwater. **A**: at hatching, **B**: 24 h after hatching, **C**: 48 h after hatching, **D**: scale (in mm): the size of the individual is 2 mm. (1) eyes, (2) yolk sac including an oil droplet, (3) pigmentation at the location of the mouth, (4) chromatophores.

following the same protocol and the same tools than previously described, during 48 h. The following day, all larvae were dead and it was not possible to continue the observations.

RESULTS

Larval development in freshwater (Fig. 1)

In freshwater, the morphological characteristics of the pro-larvae show little development, whatever the time spent in this environment (from the 48 h of storing in freshwater tanks before the beginning of experiment to the whole time of experiment in the tank 100% freshwater), leading to the conclusion that the individuals remain at the pro-larval stage (i.e., L1 stage) as long as they do not reach the sea.

Namely, the yolk sac of which the oil globule becomes proportionally prominent is never entirely absorbed, even when pro-larvae die in freshwater. At hatching, pigmentation consists in 4 chromatophores located at the mouth, on the posterior part of the yolk sac, above the digestive tract and between the anus and the caudal fin. This pigmentation tends to spread horizontally along the digestive tract with time (i.e., L1c stage). At hatching, the eyes are not pigmented. The lenses appear after 24 h, and the eye pigmentation increases progressively after ± 48 h in freshwater (i.e., L1d stage). Whatever the time spent in freshwater, mouth and anus remained close, leading to the conclusion that the pro-larvae do not feed (Fig. 1). No growth of the pro-larvae has been observed, so that they keep the same length than at hatching, namely nearly 2 mm.

In the freshwater tanks of freshwater, pro-larvae repeatedly swim towards the surface and passively sink. They also show a gregarious behaviour, the pro-larvae gathering in column of high densities in the central part of the tanks.

During the experiment, the observed mortality in the two freshwater tanks was high. In one of the two tanks, all pro-larvae died 24 h after the beginning of the experiment. In the other tank (i.e., duplicate), their survival has been recorded 1 more day.

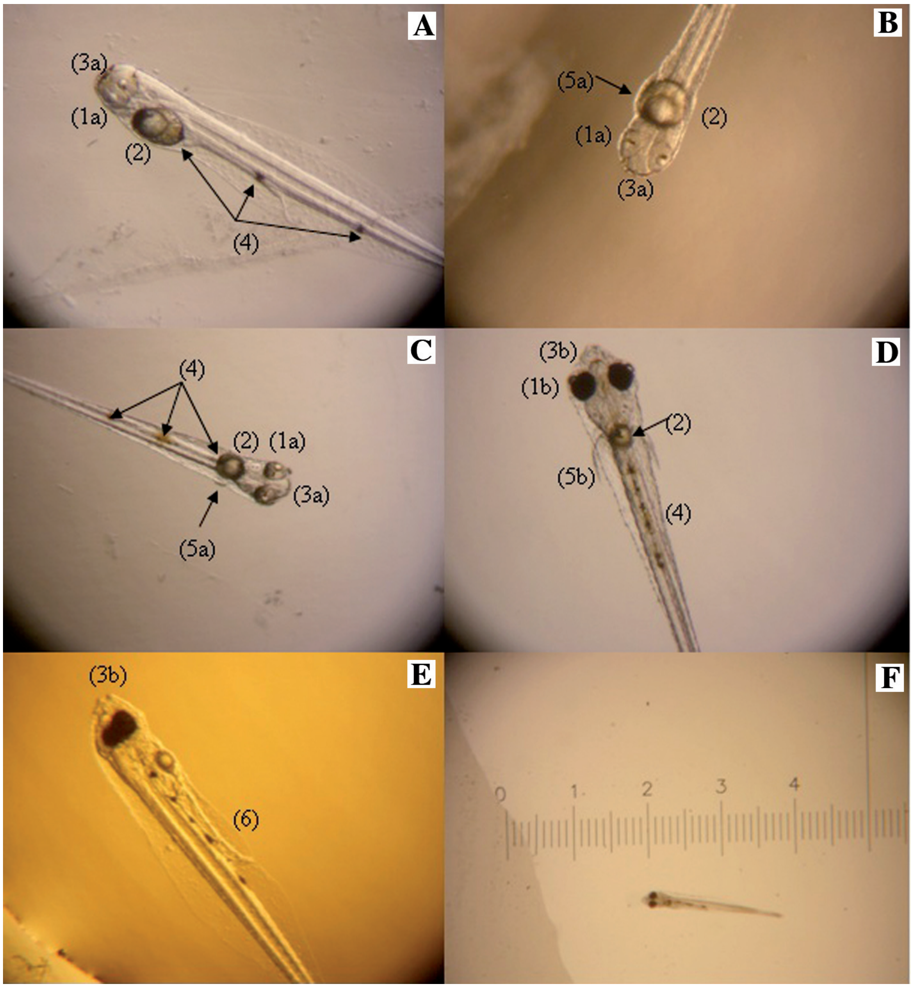

Figure 2. - Standard morphological development of *Sicyopterus lagocephalus* larvae, in seawater, after 48 h of storing in oxygenated freshwater after hatching. **A**: after 3 h in seawater, **B**: after 6 h in seawater, **C**: after 20 h in seawater, **D**, **E**: after 48 h in seawater, **F**: scale (in mm): the size of the individual is 2 mm. (1) eyes (a) translucent, (b) pigmented and functional, (2) yolk sac including an oil droplet, (3a) pigmentation at the location of the mouth, (3b) opened mouth, and shaped head, (4) chromatophores, (5a) pectoral fin buds, (5b) developed and functional pectoral fins, (6) functional digestive tract.

Standard larval development in seawater (Fig. 2)

The transfer of pro-larvae aged of 48 h (i.e., after 48 h storing in freshwater) in tanks filled with 100% seawater induces the acquisition of larvae morphological characteristics, typical of marine environment. Thus, yolk sac is rapidly absorbed until it consists in the oil globule only, after 20 h spent in seawater, corresponding to the L1d stage. Pectoral fin buds appear after 6h in seawater (i.e., L1c stage) and the fins are entirely developed and functional after 48 h (i.e., L1d stage). Pigmentation spreads above the digestive tract until it forms an almost continuous string. Eyes are entirely pigmented and functional (i.e., lenses are prominent and eyes are mobile) after ± 40 h. The mouth and anus are open after 48 h in seawater. The opening of the mouth goes with a morphological modification of the head, which appears more shaped (i.e., L2 stage) (Fig. 2). Indeed, once in the sea, after total absorption of their yolk sac, larvae feed on planktonic (phyto- and zooplankton) resources they have to catch. In parallel to the opening of the mouth and anus, the digestive tract thickens, and peristaltic movements propagate from its anterior to its posterior part. 48 h after their transfer in 100% seawater, no larval growth is recorded, so that the larvae remain at a length of nearly 2 mm.

It has to be underlined that whatever the time spent in freshwater (i.e., from immediately after hatching to 96 h of storing: that is to say as long as pro-larvae survive in this environment) before their transfer in seawater, the transformation of pro-larvae in marine larvae follows the same pattern, the same chronology and the same timing once in seawater (data not shown).

In seawater, the larvae remain on vertical position with their head oriented downward, but they also spend progressively more time on the horizontally, after the opening of the mouth. Then, after 48 h in seawater, larvae spend up to 50% of their time in a horizontal position, and show sudden accelerations in their swimming behaviour.

Influence of larval density in experimental tanks

Within the framework of this study, we tested the influence of different larval densities at the beginning of the experiment. The results show that the larval density at the time of transfer does not influence larval development (i.e., the same developmental pattern, the same chronology and the same timing were recorded whatever the initial density). The mortality being strong within the 2 days after transfer in all seawater tanks, larval densities rapidly balanced between the tanks.

Regarding these results, choosing a strong density (i.e., 100 larvae.l^{-1}) at the beginning of the experiment seems to be not prejudicial for the purpose of our study. On the contrary, it allowed to locate easily the larvae in the tanks, and facilitated their collect for observation.

Usually, in aquaculture, at the beginning of a fish larvae farming, densities never exceed 30 ind.l^{-1} (ARDA, pers. com.) in order to avoid any density dependence problems. However, *S. lagocephalus* larvae are particularly small (± 2 mm), almost totally transparent and difficult to locate in tanks. Moreover, they show a gregarious behaviour. They also necessitate a change of environment (from freshwater to seawater) to achieve their development: in terms of logistic, that particular life cycle requires rearing tanks filled up with freshwater next to tanks filled up with seawater. These constraints are not usually encountered in traditional fish farming, where larvae are bigger and exhibit simpler life-cycle (i.e., without complete change of environment).

Influence of the salinity

In the 2 tanks of 100% freshwater, *S. lagocephalus* individuals remained at the pro-larvae stage (i.e., L1a stage), as previously described. Their behaviour did not change (i.e., active upwards and passive downwards oscillations).

In all the other tanks, pro-larvae began their transformation right after their transfer, whatever the salinity in the tank. In all tanks, the larvae went through the same developmental patterns, in the same chronology, and with the same timing than previously described for the standard larval development in 100% seawater (Tab. I; Fig. 3).

Larval behaviour modifies as previously described, in all salty water tanks at the same time, with larvae spending more time in a horizontal position with sudden acceleration in their swimming behaviour.

Larval mortality was also identical in all tanks with salty water, and higher in freshwater tanks. Indeed, if pro-larvae survived only 3 to 4 days after hatching in freshwater tanks, larvae survived 7 days after hatching (i.e., 5 days after their transfer in salty water), whatever the salinity of the tanks. At the end of the 5 days of experiment, a few larvae were observed in each salty water tank, and no larvae were found alive on the 6th day. It is important to note that larvae were not fed during this experiment: starvation may explain this mortality, once the yolk sac resources are exhausted.

Concerning the pro-larvae put in the tank filled up with 6.67% of seawater: even in such a low salinity, they began to undergo the standard transformations to reach the marine larval stage L2, namely beginning of yolk sac absorption, appearance of pectoral fin buds and pigmentation evolution. Surprisingly, after their transfer back to the 100% freshwater tank, the larval modifications initiated in brackish water continued, so that 24 h after this ultimate transfer, larvae show the standard marine larvae morphological characteristics, with functional pectoral fins, opened mouth and anus, and functional digestive tract. The only notable difference concerned the yolk sac, as its absorption was strongly slowed down, but not stopped, by the transfer back in freshwater.

Table I. - Morphological modifications from the pro-larval stage (in freshwater) to the larval stage (in seawater). Pro-larvae were maintained 48 h in strict freshwater (i.e., 48 h after hatching) before their transfer in their respective tanks of seawater for the beginning of the experiment. X: appearance of new character; NO: not observed; - character already acquired before their transfer in seawater.

	100% freshwater	6.67% seawater	25% seawater	50% seawater	75% seawater	100% seawater
Body pigmentation increase	X	-	-	-	-	-
Lenses and eye pigmentation	X	-	-	-	-	-
Yolk sac resorption	NO	X (slowly)	X	X	X	X
Pectoral fin buds	NO	X	X	X	X	X
Eyes entirely pigmented and functional	NO	X	X	X	X	X
Mouth and anus open	NO	X	X	X	X	X
Morphological modification of the head	NO	X	X	X	X	X
Digestive tract thickens + peristaltic movement	NO	X	X	X	X	X
Behaviour: vertical movements	NO	X	X	X	X	X
Behaviour: horizontal acceleration	NO	X	X	X	X	X

DISCUSSION

Conditions for pro-larvae survival in freshwater: a question of time

During our experiment, we observed a strong mortality for pro-larvae in freshwater tanks, as they died 3 or 4 days after hatching. This is congruent with previous observations realised previously: for a water temperature between 20 and 23°C, the maximal survival rate was recorded between 3 and 4 days (Valade *et al*., 2009).

According to our observations, whatever the time spent in freshwater in the limit of these 4 days, before their transfer in seawater, the transformation of *S. lagocephalus* pro-larvae (i.e., L1a stage) in marine larvae (i.e., L2 stage) follows the same pattern, the same chronology and the same timing once in seawater. This observation is congruent with previous observations: Valade *et al*. (2009) showed that at 22°C, pro-larvae transferred in seawater, after 4 h and 56 h respectively in freshwater, show the same developmental rate and reach the same larval developmental stage after the same time spent in seawater. Within our study though, the chronology in the acquisition of marine characters slightly diverges from the one described in Valade *et al*. (2009), as a few characters appeared in freshwater during our observations (i.e., eye pigmentation, lens development, body pigmentation increase), whereas Valade *et al*. (2009) observed them in seawater only.

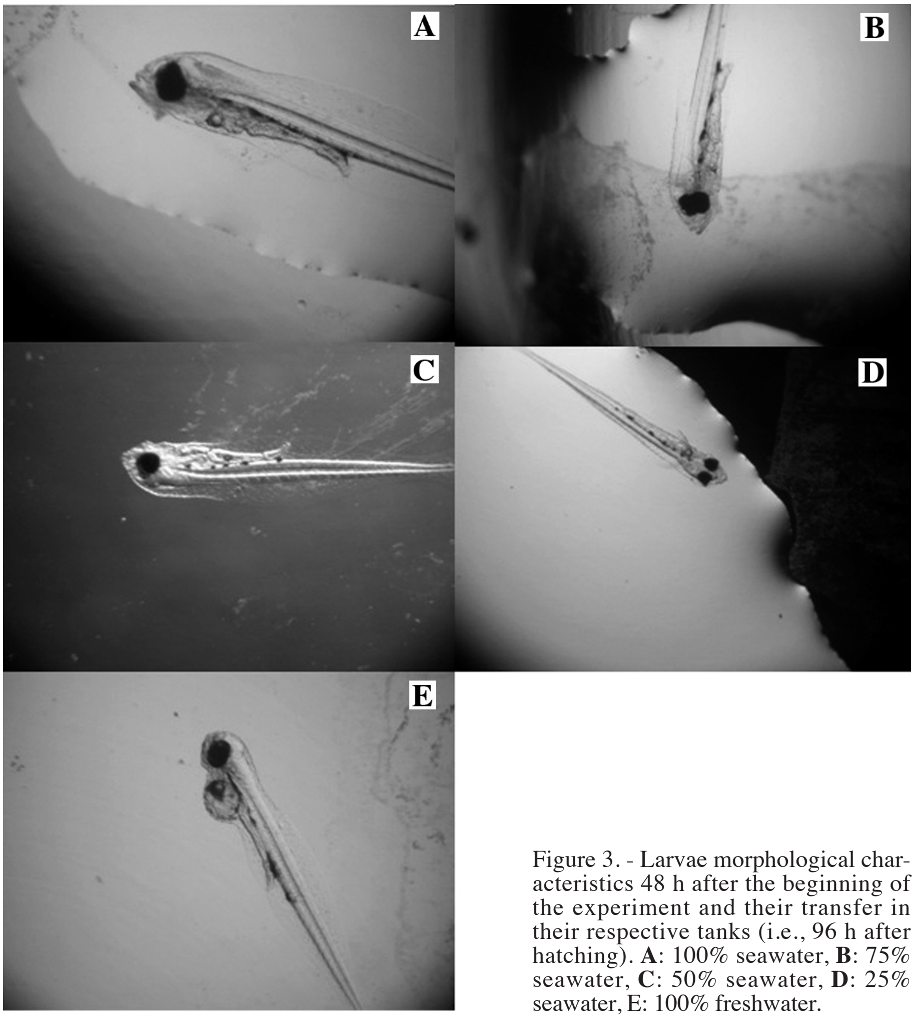

Figure 3. - Larvae morphological characteristics 48 h after the beginning of the experiment and their transfer in their respective tanks (i.e., 96 h after hatching). **A**: 100% seawater, **B**: 75% seawater, **C**: 50% seawater, **D**: 25% seawater, E: 100% freshwater.

Thus, the time spent in freshwater does not influence larval survival and development in the sea, as long as pro-larvae reach the sea alive. In Reunion Island, according to the rivers length (i.e., 36 km maximum) and the rivers flows intensity, a downstream migration duration limited to 4 days is high enough, as pro-larvae are transported downstream to the sea in less than 24 h, whatever the clutches location in the rivers (Valade *et al*., 2009). During 24 h of downstream migration, the pro-larvae abilities are sufficient to ensure their survival. Indeed, as the yolk sac begins to be absorbed once they reach the sea, it is possible that this endogeneous resource is used at the beginning of the marine larval phase only. Thus, osmoregulation changes may be the main mortality factor for *S. lagocephalus* pro-larvae in freshwater, if the downstream transport duration exceeds 4 days. However, for other amphidromous species in other regions (e.g., *Rhinogobius brunneus* (Temminck & Schlegel, 1845) in Japan (Iguchi and Mizuno, 1999) or *Rhinogobius* sp. in Japan (Tamada et Iwata, 2005)), early survival varies among populations according to the length of the river in relation with larval starvation, when their endogenous resources provided by the yolk sac are entirely absorbed (McDowall, 2009).

Because of their vertical behaviour of upward active swimming alternating with passive sinking, *S. lagocephalus* pro-larvae are supposed to be carried faster to the sea. As they can benefit of the faster surface current, they probably need less than 24h to reach the sea, from the rivers in Reunion Island. Beside, this behaviour should avoid the pro-larvae to be trapped on the bottom, which would delay or prevent their transport to the sea. This vertical behaviour is largely reported among Sicydiinae gobies, and has been observed both on the field by snorkelling and in experimental tanks, for *Sicyopterus japonicus* in Japan (Iida *et al*., 2010), in experimental tanks for *Sicydium punctatum* in Dominica (Bell and Brown, 1995), and for *Sicyopterus stimpsoni* pro-larvae in Hawaii (Kinzie, 1993). Knowing that these different Sicydiinae species are characterised by poorly developed freshwater pro-larvae stage, it seems crucial that they reach the sea as fast as possible to pursue their development. In particular, eyes seem to be not functional during the freshwater phase, even though a beginning of pigmentation was observed in freshwater during this study. The absence of functional eyes and their weak ontogenetic development make the pro-larvae easy preys, as they seem unable to avoid their predator.

According to these different aspects, it seems logical that any obstacle trapping pro-larvae in the river and preventing them from reaching the sea in less than 4 days is prejudicial for the pro-larvae survival and thus, for the species survival.

Influence of salinity on the life cycle of amphidromous gobies

Our study shows that even a very low salinity (i.e., 6.67% of sea-water in a 45 l tank corresponding to volumes of 3 l of seawater and 42 l of freshwater) is sufficient to induce the transformation of pro-larvae (i.e., L1 stage) into larvae (i.e., L2 stage). So, it seems that we did not underline a minimum salinity threshold to induce the acquisition of marine larvae morphological characteristics: as soon as pro-larvae reach brackish water -even at low salinity- they begin their transformation. Moreover, if the pro-larvae, after a first contact with brackish water, secondarily return to freshwater, their transformation is not stopped. This result raises a new question: is there a minimum duration to spend in salty water to induce larval transformation?

Beside, if larvae can continue their transformation in freshwater, as long as they previously spent enough time in brackish water, do they have to go through such a long dispersive marine phase or is it possible that *S. lagocephalus* larvae remain in estuaries before migrating back to rivers to achieve their life-cycle? Genetic studies revealed a high gene flow and genetic homogeneity for *S. lagocephalus* within Reunion Island and with other populations from the South-West Indian Ocean, namely Mauritius Island or Indo-Pacific (Lord, 2009; Lord *et al*., in press). Allochthonous recruitment was also highlighted at the Indian Ocean scale, implying individuals from Mayotte (Comoros) (Lord, 2009; Lord *et al*., in press), but also probably sporadically from the western Indonesian region or other location from the Indian Ocean (Madagascar or India) (Lord, 2009). These genetic insights let no doubts about a dispersive marine phase for this amphidromous species with such a widespread distribution. Regarding their life history traits and their ecology, a long oceanic larval dispersive stage maximises their chances to find a suitable habitat, knowing the particularities of insular river systems (i.e., oligotrophic rivers recently formed, subject to extreme climatic and hydrological variations on a seasonal scale (Keith, 2003)). Identical experiments should be conducted on Mascarene endemic species (e.g., *Cotylopus acutipinnis*), in order to assess their tolerance to salinity changes, and eventually their ability to remain in estuaries or close to the insular systems where they come from (Keith *et al*., 2005b).

Iida *et al*. (2010) also showed that adults, larvae and eggs of another Sicydiinae species, *Sicyopterus japonicus* (Tanaka, 1909), revealed to be more tolerant to salinity changes than commonly assumed. Indeed, even though the adults of this species were thought to survive only in freshwater, Iida *et al*. (2010) showed that they surprisingly survive also in brackish water (1/3 seawater), and therefore they may survive in estuaries and lower parts of the rivers, while strict seawater remains lethal. Similarly, eggs were assumed to hatch only in freshwater, but the hatching rate was almost the same in brackish (i.e., 1/3 seawater) treatments, while lower in strict seawater, and larvae need also higher salinity to develop (Iida *et al*., 2010). The pattern of salinity tolerance is a bit different for *Rhinogobius sp*. in Bonin Islands, with larvae surviving longer in brackish water (50% seawater) or freshwater, while seawater induces stronger mortality (Yokoi et Hosoya, 2005). Salinity tolerance is an interesting subject to study for amphidromous species as they need to change of environments twice to accomplish their life-cycle, and that raises questions about their evolutionary pattern.

Conclusion

This study brings new insights into salinity tolerance of an amphidromous goby, *S. lagocephalus*, leading to the conclusion that this species needs salinity changes in its environment to carry out each phase of its life cycle, without being strict upon the salinity value itself. In particular, for the transformation of the freshwater pro-larvae (i.e., L1 stage) into marine larvae (i.e., L2 stage), a large range of salinities lead to the same result, with the same timing. However, this study raises new questions and need to be deepened. Is there a minimum duration to spend in salty water to induce the acquisition of marine larvae morphological characteristics? Is this transformation of freshwater pro-larvae into marine larvae a real metamorphosis (*i.e*. implying specific hormones)? Indeed, the need of salty water to induce larval transformation leads to the assumption that ionic pumps may be involved in this process (Taillebois *et al*., 2011). We also wonder what the behaviour of larvae at sea is during their pelagic dispersal phase and if it explains their large distribution.

This study confirms previous works aiming at characterising pro-larval and larval stages (Teichert, 2009; Valade *et al*., 2009), with some discrepancies, for example for the eye pigmentation which begins in freshwater within this study, and only in salty water for the other study (Valade *et al*., 2009). However, the main conclusions of these different studies on *S. lagocephalus* in Reunion Island, are in agreement with other studies on amphidromous gobies in other regions (e.g., Iida *et al*., 2010). In all cases, the freshwater pro-larvae need to reach the sea in a narrow temporal scale (e.g., within 4 days in the case of *S. lagocephalus* in Reunion Island) otherwise they die, either of starvation (e.g., *S. japonicus*) or probably of physiological causes (i.e., osmoregulation) but these have to be confirmed for *S. lagocephalus* in Reunion Island. In this context, any obstacle in the river/sea corridor (e.g., exsiccation periods downstream of the rivers), delaying their arrival to the sea, is lethal for the pro-larvae and prejudicial for the wealth of the stock, already damaged by over-fishing on recruiting post-larvae.

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